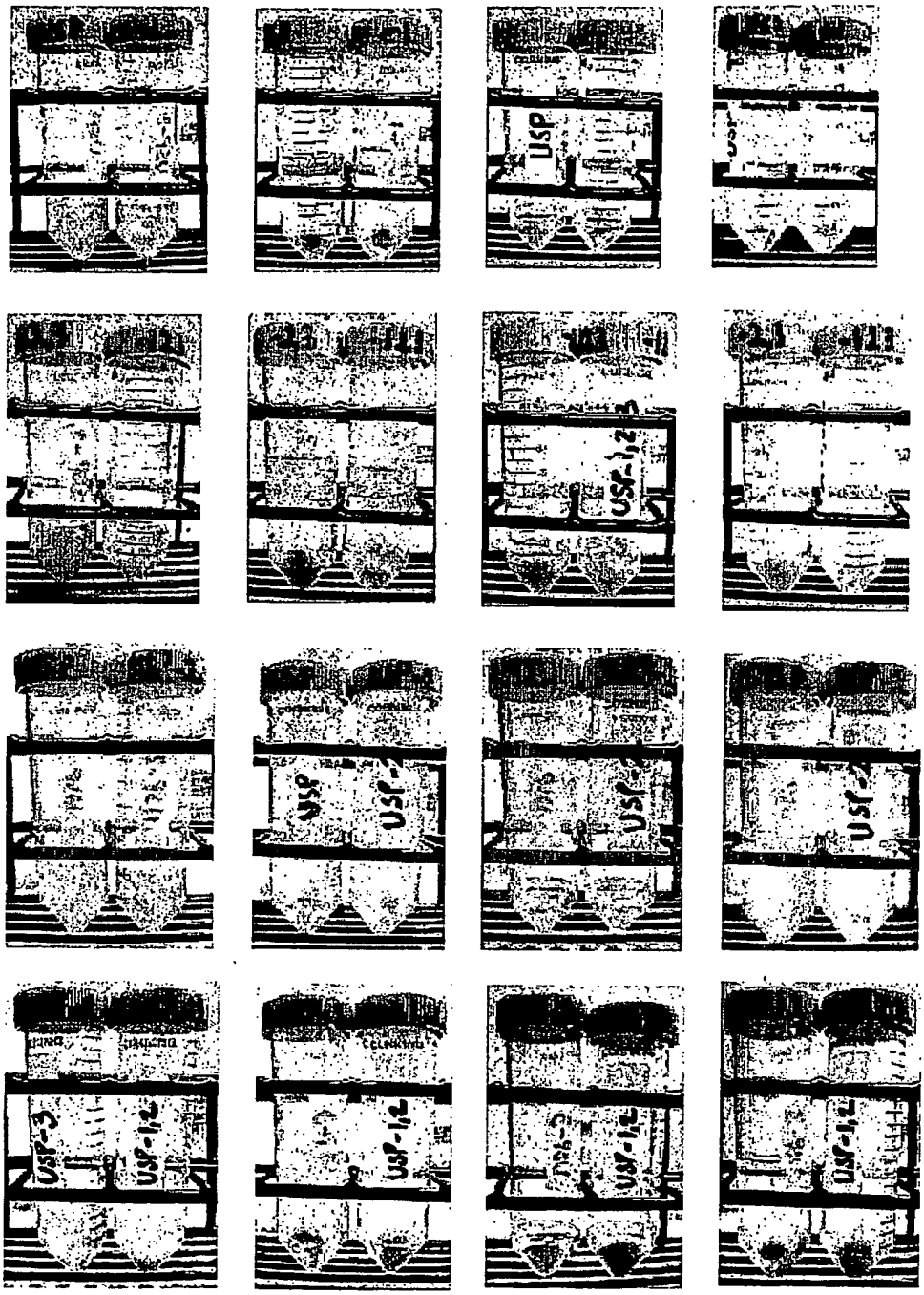
Fig.1: Effect of variation in composition of USP solution on the processing of sputum specimens.



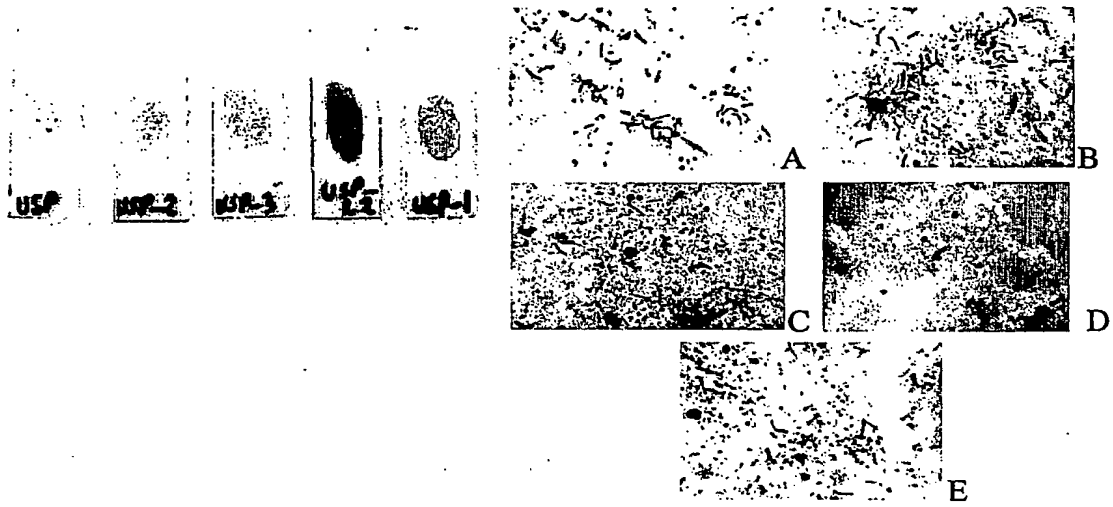
Step 1: Homogenization of the specimen by adding the processing solution

Step 2: Pellet obtained after step 1

Step 3: Pellet obtained after second wash with the processing solution.

Step 4: Pellet obtained after water wash which is ready for smear microscopy, culture and DNA isolation from PCR.

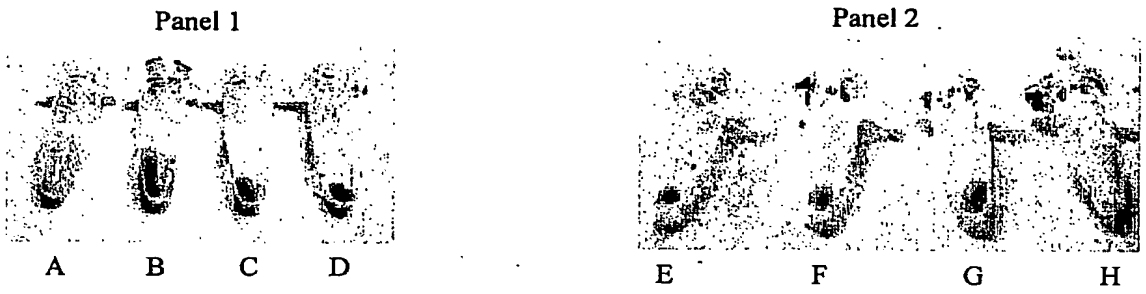
Fig.2: Effect of variation of USP solution composition on smear microscopy of sputum specimens. The slides and the corresponding fields are shown.



USP solution without GuHCl.(USP-1)

A: USP solution; B: USP solution without sarcosyl (USP-2); C: USP solution without β-mercaptoethanol (USP-3); D: USP solution without GuHCl and sarcosyl (USP-1,2); E: USP solution without GuHCl.(USP-1) (Magnification:1200X)

Fig. 3 a. Pellets obtained for PCR after processing sputum specimen using USP solution of varying compositions



A: USP solution without GuHCl, sarcosyl and β-mercaptoethanol (USP-1,2,3)

B: USP solution without sarcosyl and β-mercaptoethanol (USP-2,3).

C: USP solution without GuHCl (USP-1).

D and E: USP solution.

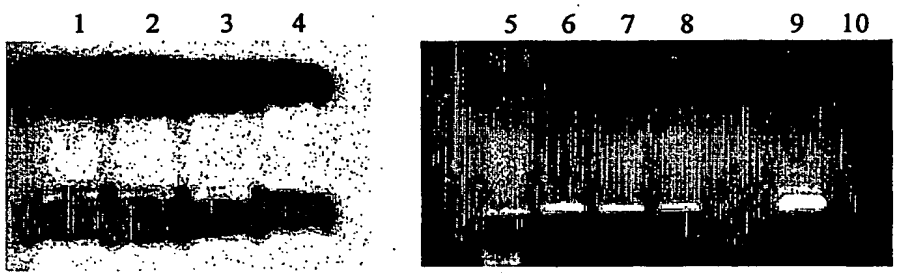
F: USP solution without sarcosyl (USP-2)

G: USP solution without β-mercaptoethanol (USP-3)

H: USP solution without GuHCl and sarcosyl (USP-1,2)

Panel 1 and Panel 2 represent two different sputum specimens of different physical characteristics.

Fig. 3b. IS6110 PCR profile of the *M. tuberculosis* DNA isolated from the sputum specimens processed by the different variations of USP solution



1: USP solution without GuHCl and sarcosyl (USP-1,2)

2: USP solution without GuHCl and β-mercaptoethanol (USP-1,3)

3: USP solution without sarcosyl and β-mercaptoethanol (USP-2,3).

4: USP solution without GuHCl, sarcosyl and β-mercaptoethanol (USP-1,2,3)

5: USP solution without GuHCl (USP-1).

6: USP solution.

7: USP solution without sarcosyl (USP-2)

8: USP solution without β-mercaptoethanol (USP-3)

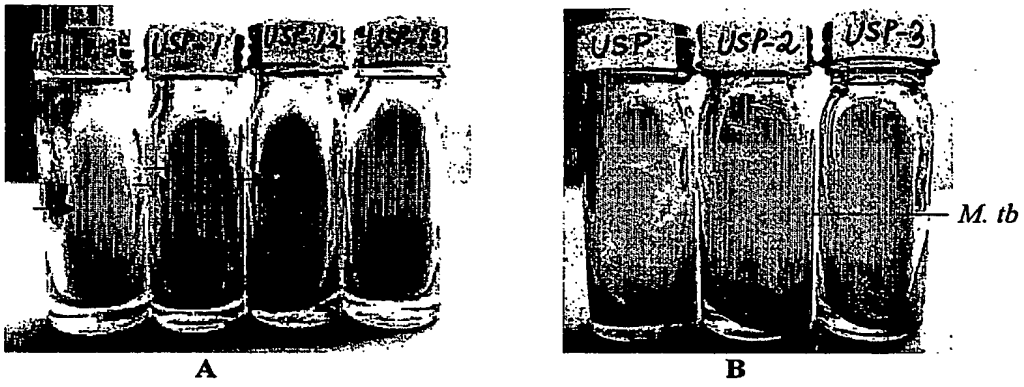
9: DNA positive control

10: DNA negative control

Lane 4 shows complete inhibition of PCR and all the lanes except for lane 6 shows partial inhibition of PCR.

Fig 4.

Effect on culture of sputum specimen on LJ medium processed by different variations of USP solution deprived of single or multiple components.



A: The LJ slants from left to right shows cultures obtained from a sputum specimen processed by different variations of the USP solutions not containing GuHCl. The sputum specimen (obtained by pooling three AFB positive sputa) was divided into equal aliquots and processed by the following solutions:

(USP- 1,2,3): USP solution without GuHCl (1), sarcosyl (2) and β-mercaptoethanol (3). Contains only Tris-HCl and EDTA.

(USP-1): USP solution without GuHCl (1). Contains sarcosyl (2), β-mercaptoethanol (3), Tris-HCl and EDTA.

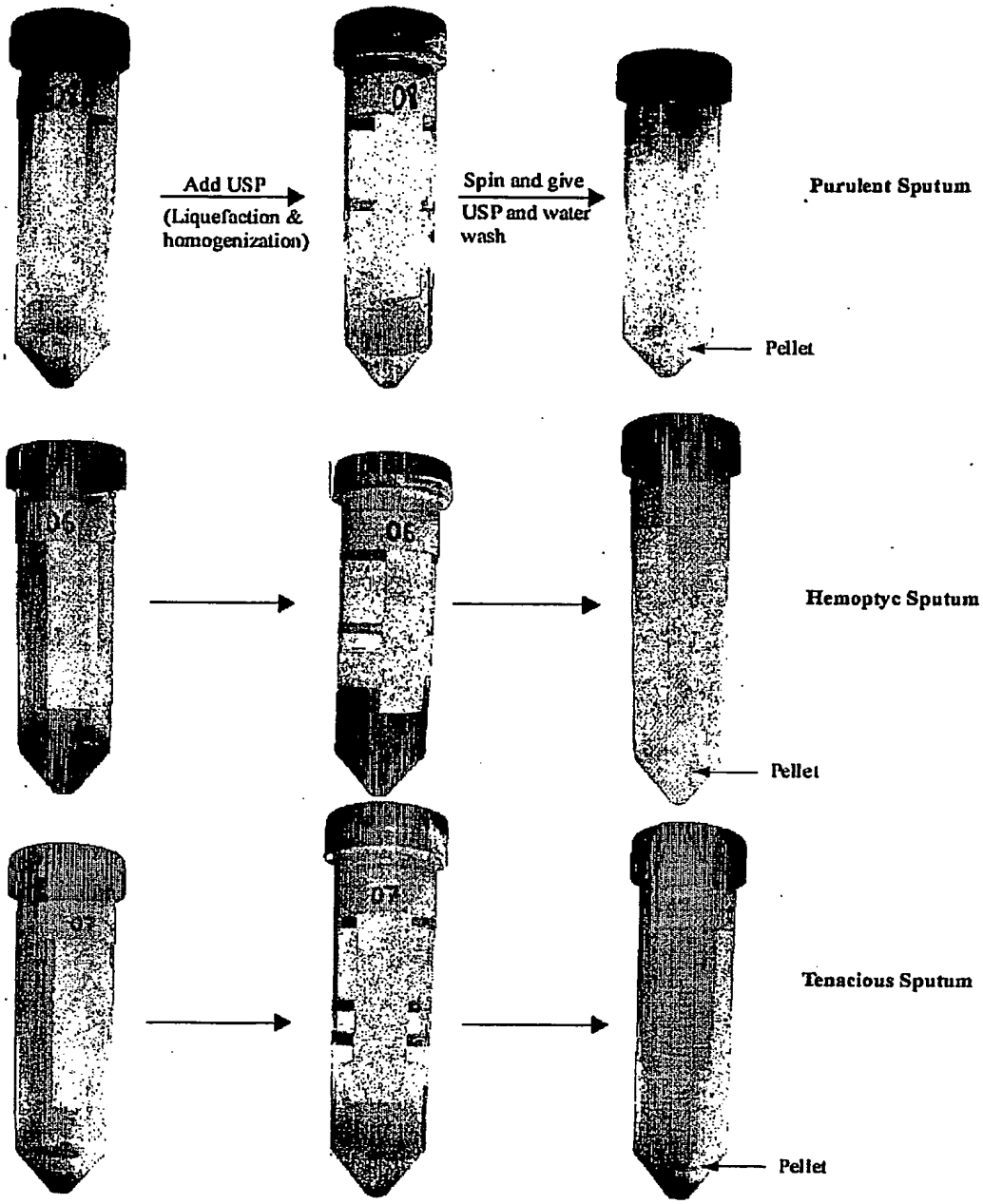
(USP- 1,2): USP solution without GuHCl (1) and sarcosyl (2). Contains only β-mercaptoethanol (3), Tris-HCl and EDTA.

(USP- 1,3) USP solution without GuHCl (1) and β-mercaptoethanol (3). Contains only sarcosyl (2) Tris-HCl and EDTA.

The slants were photographed after a three week culture period. Each slant showed varying degrees of contamination (indicated by arrows ⟶) proving that GuHCl had a crucial role to play as a decontaminant in processing clinical specimens.

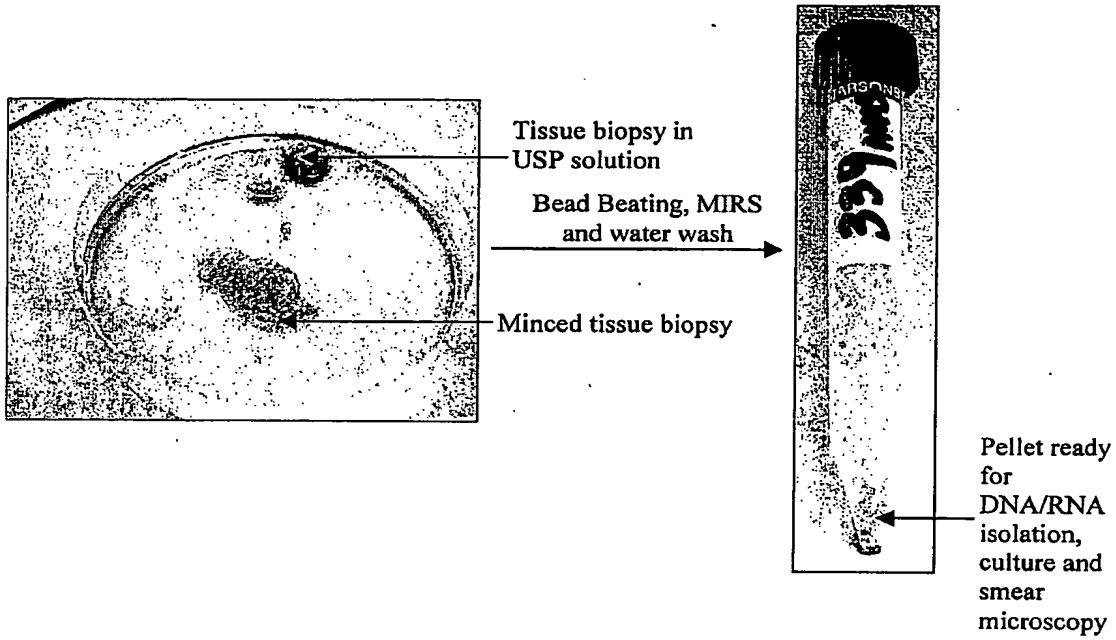
B: No contamination was detected when the specimen was processed with the USP solution and variations of the USP solution containing GuHCl but deprived of β-mercaptoethanol or sarcosyl (USP-2, USP-3). All the cultures were positive for mycobacterium at the end of three weeks.

Fig. 5a. **Effect of USP solution on sputum with different physical characteristics**



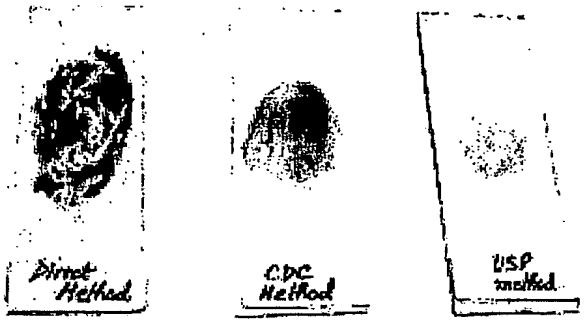
(A) Sputum of varying characteristics was mixed with USP solution leading to (B) liquefaction and decontamination. After centrifugation to remove cell debris, denatured protein and other contaminants, the pellet (C) was obtained.

Fig.5b: Effect of USP solution on tissue biopsy material



The biopsy material is softened by digestion with USP solution and can be subjected to subsequent processing for smear microscopy, DNA/RNA isolation and culture by the USP method.

Fig. 6.



A B C

Physical appearance of the smear prepared from the same sputum sample processed by direct, CDC and the USP smear method. (A) One loopful of purulent portion of a sputum sample was taken in a 3 mm sterile wire loop and smeared on a glass slide. (B) An equal aliquot of the same sputum sample was processed by the CDC method and two loopfulls was smeared on glass slide. (C) A third equal aliquot of the same sample was processed by the USP method an 10 % of the processed pellet was smeared on a glass slide.

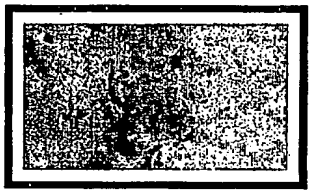
Fig. 7.

~10^7 AFB/ml
3+

~10^6 AFB/ml
3+

~10^5 AFB/ml
3+

10^4 AFB/ml
2+



3270 AFB/ml
1+

310 AFB/ml
Scanty (7 AFB/100 oil immersion fields)

Determination of limit of detection of USP smear microscopy: A COPD sputum sample was confirmed to be AFB negative by smear, PCR and culture. It was then homogenized and divided into equal aliquots which were spiked with serial dilutions of a logarithmic-phase culture of *M. tuberculosis* H37Rv. Each spiked aliquot was processed by the USP method and 10% was smeared, stained by ZN method and visualized under oil immersion lens. Magnification: 6,000 X. (1+ and scanty slides: 8,000X).

Fig. 8.

Conversion of negative samples to positive by USP method of smear microscopy:

Two sputum samples collected from DOTS centre at NDTBC, which were negative by direct method of smear microscopy turned into 2+ and 3+ respectively by USP method of smear microscopy. 10% of the processed sputum pellet by the USP method was smeared and stained by ZN method and visualized under oil immersion lens.

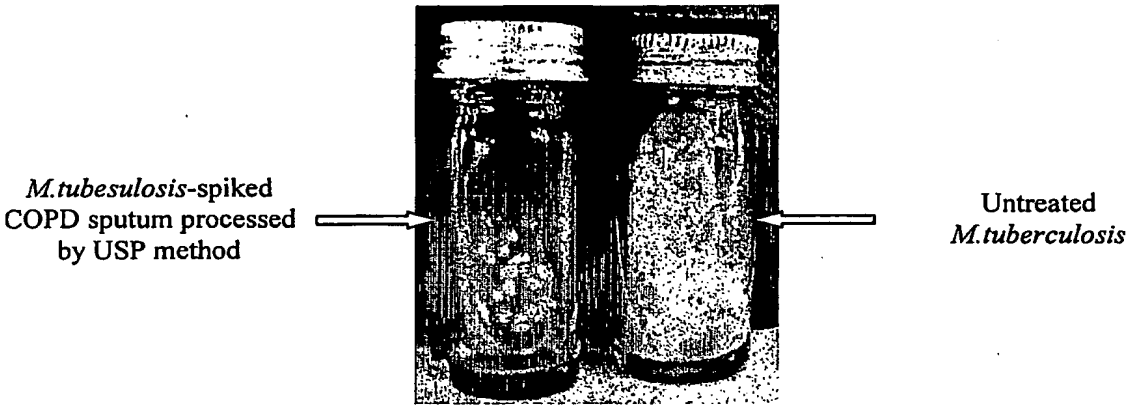
Magnification: 8,000 X

Fig. 9.

DIRECT (1+) USP (3+) CDC (2+)

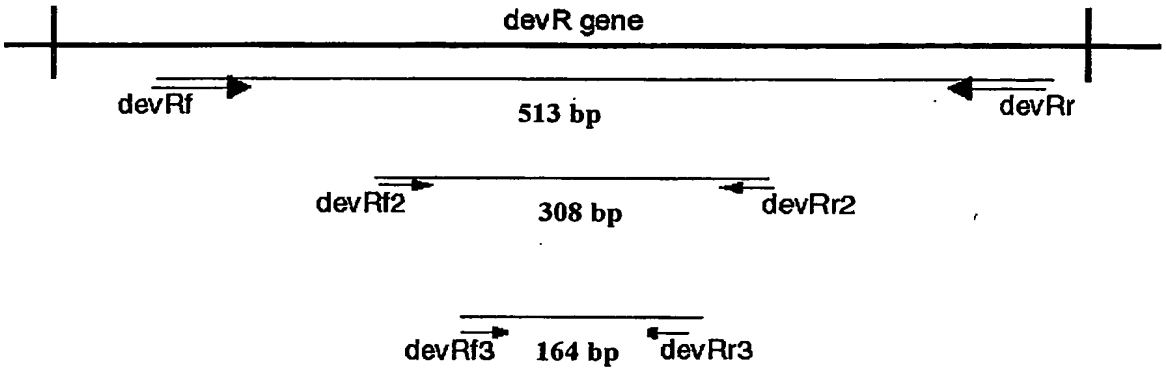
Enhancement in smear status by USP method of smear microscopy as compared to the direct and CDC methods of smear microscopy: The same sputum sample was prepared for smear microscopy by the direct, CDC and USP methods and stained by ZN method and visualized under oil immersion lens. Magnification: 6,000 X

Fig. 10.



Effect of effect of USP solution treatment on the viability of *M. tuberculosis*: A COPD sputum sample was first confirmed to be AFB negative by smear, PCR and culture. It was spiked with logarithmic-phase culture of *M. tuberculosis* H37Rv and the samples processed by the USP method. Fifty percent was inoculated on LJ medium. Fifty percent of a untreated duplicate untreated aliquot of *M. tuberculosis* H37Rv was inoculated as such on LJ medium. They were incubated at 37^0C and monitored at weekly intervals.

Fig. 11.

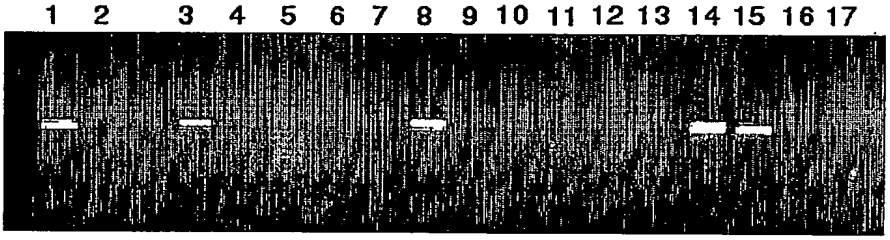


Primers for amplification of *devR* gene of *M. tuberculosis*. 513 bp, 308 bp and 164 bp are the sizes of the amplification products obtained with the three primer pairs indicated.

Fig. 12.

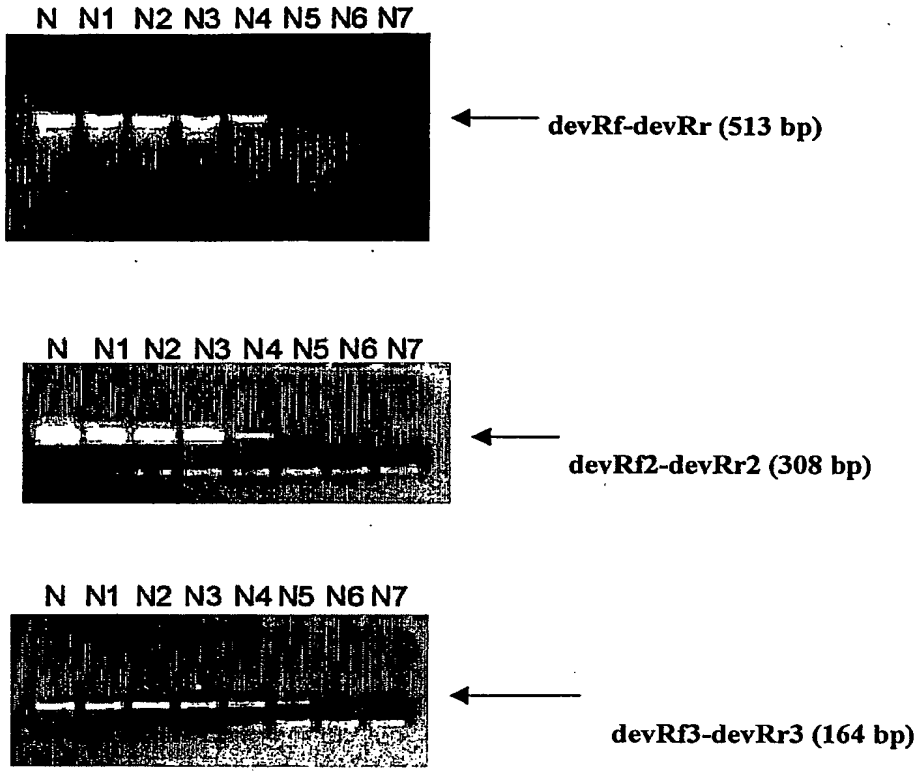
PCR amplification products obtained with DNA extracted from different *Mycobacterium sp.* using the primer pairs devRf3 and devRr3. Lane 1: *Mycobacterium africanum*, Lane 2: *M. avium*, Lane 3: *M. bovis*, Lane 4: *M. fortuitum*, Lane 5: *M. gordonae*, Lane 6: *M. intracellulare*, Lane 7: *M. kansasi*, Lane 8: *M. microti*, Lane 9: *M. phlei*, Lane 10: *M. scrofulaceum*, Lane 11: *M. smegmatis*, Lane 12: *M. xenopi*, Lane 13: *M. vaccae*, Lane 14: *M. tuberculosis* H37Rv, Lane 15: *M. tuberculosis* clinical isolate., Lane 16: PCR

Fig. 13.



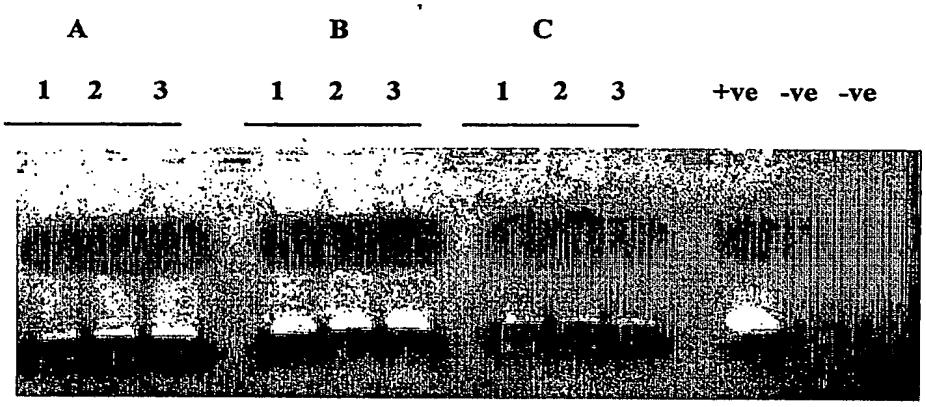
PCR amplification products obtained with DNA extracted from different *Mycobacterium sp.* using the primer pairs devRf2 and devRr2. Lane 1: *Mycobacterium africanum*, Lane 2: *M. avium*, Lane 3: *M. bovis*, Lane 4: *M. fortuitum*, Lane 5: *M. gordonae*, Lane 6: *M. intracellulare*, Lane 7: *M. kansasi*, Lane 8: *M. microti*, Lane 9: *M. phlei*, Lane 10: *M. scrofulaceum*, Lane 11: *M. smegmatis*, Lane 12: *M. xenopi*, Lane 13: *M. vaccae*, Lane 14: *M. tuberculosis* H37Rv, Lane 15: PCR negative control.

Fig. 14.



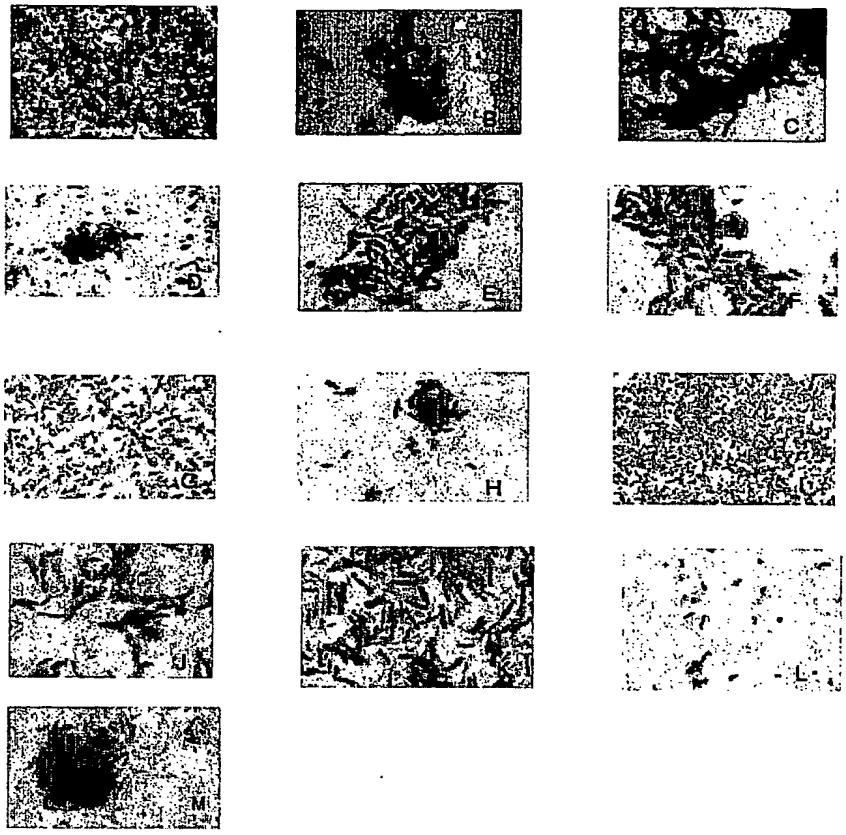
PCR amplification profiles of serial dilutions of *M. tuberculosis* DNA using three different primer pairs targeting the *devR* gene. In each panel the dilutions are as follows N: Neat, N1: 1:10, N2: 1:100, N3: 1:1000, N4: 1:10000, N5: 1:100000, N6: 1:1000000, N7: 1:10000000

Fig. 15



Comparison of PCR amplifiable *M. tuberculosis* DNA isolation from sputum by lysis using solution 3, solution 3 with 0.01% Triton X-100 and lysis reagents (Solution A, B and C). : Each panel (A, B and C) shows IS6110 amplification obtained from three different sputum samples. In each panel; Lane 1: Lysis using solution 3 alone; Lane 2: Lysis using solution 3 with 0.01% triton X-100 and Lane 3: Lysis using lysis reagents (Solution A, B and C). +ve and –ve indicate positive control and negative control PCR reactions, respectively.

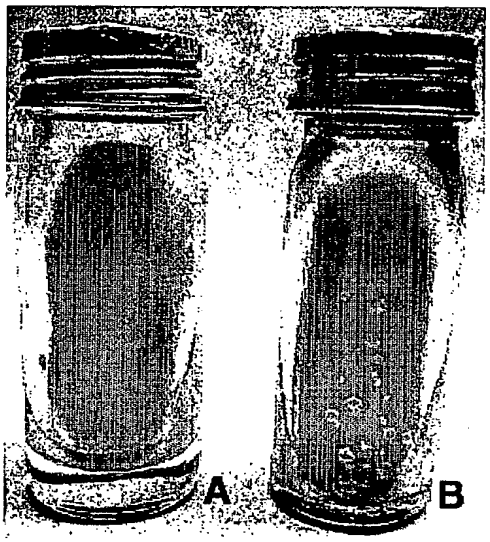
Fig. 16: Smear microscopy of USP-treated mycobacteria other than *M. tuberculosis* (MOTT).



Mycobcterial species other than *M. tuberculosis* were treated with USP solution for 10 minutes followed by water wash. They were then subjected to Ziehl-Neelsen staining, culturing on LJ medium and DNA isolation for PCR. The ZN staining of the following mycobacterial species are shown in the figure:

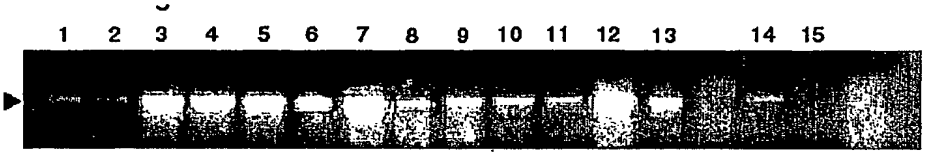
A: *Mycobacterium africanum*, B: *M. avium*, C: *M. bovis* (BCG), D: *M. bovis*, E: *M. fortuitum*, F: *M. gordonae*
G: *M. intracellulare*, H: *M. kansasi*, I: *M. microti*, J: *M. scrofulaceum*, K: *M. smegmatis*, L: *M. vaccae*
M: *M. phlei*.

Fig.17: Culture of MOTT bacilli after USP treatment



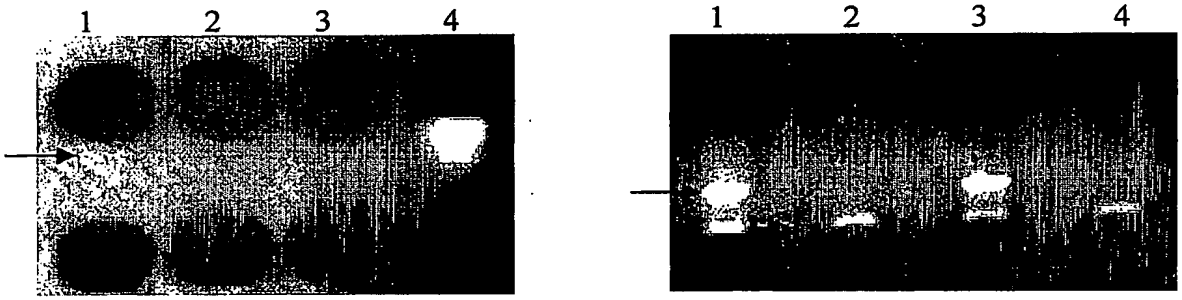
Fourteen different mycobacterial species were treated with USP solution for 10 minutes, centrifuged and washed once with sterile. A loopful of USP-treated cells were inoculated on LJ medium. A: *M. gordonae*, and B: *M. fortuitum* colonies obtained after three days.

Fig. 18: Amplification of DNA isolated from USP-treated MOTT bacilli



Fourteen different mycobacterial species were treated with USP solution for 10 minutes, centrifuged and washed once with sterile water. The pellets were subjected to DNA isolation by boiling with 0.1% Triton X-100 and subjected to PCR amplification targeting the 65 KD heat shock protein gene of *M. tuberculosis*
Lane 1: *Mycobacterium africanum*, Lane 2: *M. avium*, Lane 3: *M. bovis*,
Lane 4: *M. bovis* BCG, Lane 5: *M. fortuitum*, Lane 6: *M. gordonae*,
Lane 7: *M. intracellulare*, Lane 8: *M. kansasi*, Lane 9: *M. microti*, Lane 10: *M. phlei*,
Lane 11: *M. scrofulaceum*, Lane 12: *M. smegmatis*, Lane 13: *M. vaccae*,
Lane 14: *M. tuberculosis*, Lane 15: DNA negative control.

Fig. 19: **RT-PCR reaction with *M. tuberculosis* RNA isolated from sputum.**

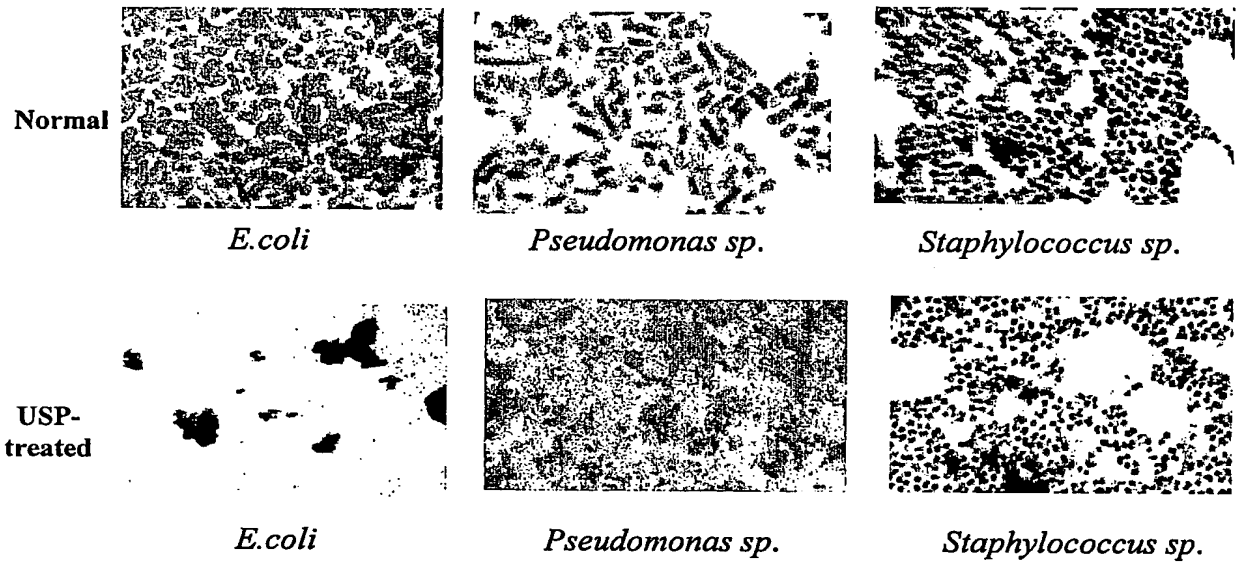


Panel A Panel B

Panel A: Amplification profile of the *Rv3134c* mRNA RT-PCR. Lane 1: RT positive, lane 2: RT negative, Lane 3: DNA negative control, Lane 4: DNA positive control.

Panel B: Amplification profile of the 23s rRNA RT-PCR. Lane 1: RT positive, lane 2: RT negative, Lane 3: DNA positive control, Lane 4: DNA negative control.

Fig. 20: Smear microscopy of USP-treated Gram positive and Gram-negative bacteria



Magnification: 1200X

E. coli and *Pseudomonas sp.* lose their morphology on being treated with USP solution. *Staphylococcus sp.* remains unaffected.

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